8

Preservation and production of starter cultures

8.1 Introduction

The manufacture of yoghurt is now more centralised than in the past and while successful production is directly related to the processing techniques employed, the correct selection, preservation, handling and propagation of the starter cultures helps to standardise and maintain uniformity in the quality of the end product.

Yoghurt cultures consist of two species (i.e. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) and as these organisms are mainly grown and propagated together, they are referred to as mixed strain starter cultures. The culture organisms are preserved in small quantities known as stock cultures. When these cultures are reactivated for use in the dairy, a scale-up system of propagation is employed to supply the required volume. For example, if the daily production of yoghurt is 25 000 l and rate of inoculation is 2 ml 100 ml^{-1}, then the amount of starter needed is 500 l. Therefore the various stages of propagation are:

$$\text{Stock} \xrightarrow[1\,\text{g or ml}]{} \text{Mother} \xrightarrow[20\,\text{ml}]{} \text{Feeder} \xrightarrow[10\,\text{l}]{} \text{Bulk} \xrightarrow[500\,\text{l}]{} \text{Processing vat}$$

The stock and mother cultures are propagated in the laboratory, while the feeder and bulk cultures are produced in the starter room of the dairy. The above stages of culture propagation are illustrated in Fig. 8.1.

An active bulk starter culture must have the following characteristics:

- It must contain the maximum number of viable cells.
- It must be free from any contaminants, e.g. coliforms or yeasts and moulds.
- It must be active under processing conditions in the dairy and hence maintenance of the intermediate and other cultures is extremely important.

The mother and feeder cultures are grown in sterile media, mainly milk, under aseptic conditions and the activity of such cultures can be maintained by applying one of the following approaches (Foster, 1962). First, reducing or controlling the metabolic activity of the organisms by ordinary refrigeration; this is for short-term storage of a starter culture and it can be kept viable for up to a week. Second, con-

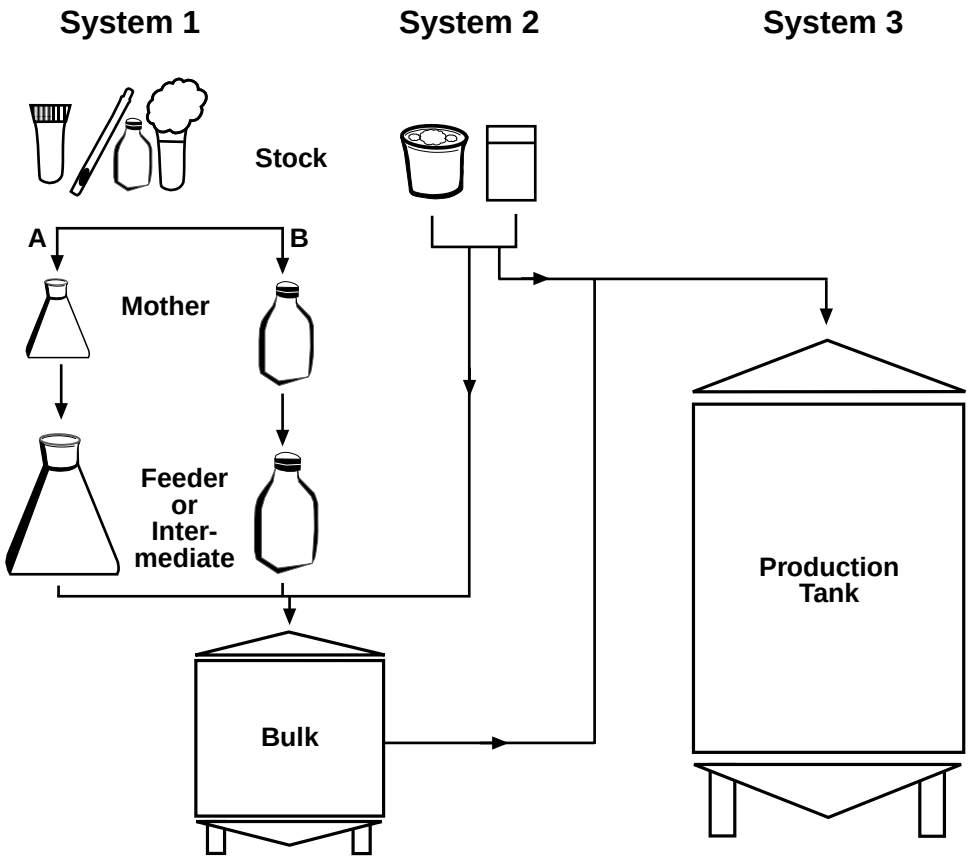

Fig. 8.1 Starter culture preparation

Note: System 1: stock culture may be liquid, freeze dried or frozen at –196°C for the production of bulk starter. Systems 2 & 3: stock culture may be concentrated freeze dried or frozen at –60°C to –196°C for the production of bulk starter and yoghurt, respectively.

centration and separation of the organisms from their wastes, followed by resuspension in a sterile medium and finally preservation by drying or freezing (Tamime and Robinson, 1976; Robinson, 1983; Tamime, 1990). The latter forms are used for extended storage of the starter bacteria and such cultures may be obtained from stock collections available in dairy research establishments, colleges or culture bank organisations, or from commercial starter manufacturers.

8.2 Methods of starter culture preservation

It is essential that starter cultures are preserved in order to maintain an available stock of these micro-organisms for the production of bulk starter and, in the case of a starter failure, some types of preserved cultures could be used for direct-to-vat inoculation (DVI). Also, successive culture transfers or subculturing can induce mutants which may alter the overall behaviour and general characteristics of the starter. Furthermore, in the case of mixed starter cultures, successive subculturing

could alter the balance or ratio of *S. thermophilus*:*L. delbrueckii* subsp. *bulgaricus*; in "bio" starters the counts of *Lactobacillus acidophilus* and *Bifidobacterium* spp. will be altered.

In general, dairy starter cultures may be preserved by one of the following methods:

- Liquid starter.
- Dried starter: (a) unconcentrated (spray dried or freeze dried/lyophilised; these methods are rather old and not used at the present time), and (b) concentrated freeze dried.
- Frozen starter: (a) frozen at –20°C (unconcentrated), (b) deep frozen at –40°C to –80°C (concentrated), and (c) ultra low temperature freezing at –196°C in liquid nitrogen (concentrated).

It can be observed that the main methods of starter culture preservation involved concentration of the bacteria, as well as various techniques of drying and freezing, and hence, the viability of a preserved culture may be dependent on:

- the basic growth medium,
- the presence of cryoprotective agents,
- rapid removal of metabolic compounds, e.g. lactic acid and carbonyl compounds,
- the nature of the suspending medium (if employed),
- conditions of freezing and/or drying,
- rate of thawing (deep frozen cultures),
- methods of concentration.

The latter aspect, sometimes referred to as cell biomass concentration, is of great importance; the number of bacterial cells per unit weight or volume is measured by counting the number of colonies produced after serial dilution, on an agar medium and the results are recorded by colony forming units (cfu) ml^{-1} or g^{-1}. However, the cell biomass can be concentrated using different systems. For further details refer to Section 8.3.2.

Nevertheless, the starter bacteria subjected to these physical conditions may die or be injured and, in view of the economic importance of starter cultures in the dairy industry, the general aim of scientists in this field has been to minimise the death rate of, or injury to, the preserved cultures. For further information about the factors affecting the survival of micro-organisms, including dairy starter cultures, reference may be made to Gray and Postgate (1976), Andrew and Russell (1984) and Hurst and Nasim (1984).

8.2.1 Liquid starters

Starter cultures can be preserved in a liquid form using one of two different growth media. The first type is reconstituted skimmed milk powder (SMP) (10–12 g $100\,g^{-1}$ SNF (solids-not-fat)) which is free from antibiotics. The milk is sterilised by autoclaving at 69–103 kPa or 121°C for 10–15 min, and a sample is incubated for a week at 30°C to check its sterility. After inoculation (1 or 2 ml $100\,ml^{-1}$), the milk is incubated at 30°C for 16–18 hours or at 42°C for 3–4 hours. At the end of the incubation period, the clotted culture must be cooled immediately and it can then be stored for up to a week at ordinary refrigeration temperature (e.g. <10°C). Personal experience suggests that if the acidity of the cold culture is around 0.85 g $100\,g^{-1}$ lactic

acid, both the activity of the starter and the ratio of *S. thermophilus* to *L. delbrueckii* subsp. *bulgaricus* (1:1) are easily maintained. This type of starter culture is referred to in the industry as a working stock culture. Alternatively, cool, autoclaved milk may be inoculated with a starter culture and then stored under refrigeration for incubation whenever it is required. It is worthwhile pointing out that successive subculturing is labour intensive, expensive and can induce mutant strains; furthermore, trained personnel are required to perform such duties in the laboratory. A maximum limit of 15–20 subcultures is recommended for the yoghurt starter bacteria to safeguard the proper ratio between cocci and the rods, and to reduce the effect of mutation.

A slightly extended preservation of liquid cultures (i.e. reserved stock culture) can be achieved using litmus milk [($g\,100\,g^{-1}$) reconstituted SMP 10–12, litmus solution 2, yeast extract 0.3, dextrose/lactose 1; enough calcium carbonate to cover the bottom of the test tube; panmede 0.25 and lecithin 1, and both adjusted to pH 7]. The medium is autoclaved at 69 kPa for 10 min and incubated for a week to check sterility (Shankar, personal communication). The inoculated medium is incubated for a short period of time (42°C for 12 hours), and stored under ordinary refrigeration; it is only necessary to reactivate the culture once every 3 months. However, Kang *et al*. (1985) preserved liquid cultures in the presence of $CaCO_3$ and found, for example, *L. acidophilus* remained active for 150 day at 37°C when the growth medium was supplemented with $1.5\,g\,100\,g^{-1}$ $CaCO_3$, whilst yoghurt cultures were preserved for 150 day at 0°C with added $CaCO_3$ ($0.5\,g\,100\,g^{-1}$). Alternative methods for the preservation of liquid starter are given: (a) cultures can be preserved for 12 months at 4°C using Na-citrate or K-phosphate buffer solutions (Sultan *et al*., 1987), (b) the activity and viability of liquid concentrated cultures of *L. delbrueckii* subsp. *bulgaricus* were improved when the cells were grown in a medium supplemented with Span 80 and then stored in $0.1\,g\,100\,g^{-1}$ Na-ascorbate after bubbling nitrogen into the culture (Kaneko *et al*., 1987), and (c) *L. delbrueckii* subsp. *bulgaricus* has been preserved successfully in gelatin spherules ($20\,g\,100\,g^{-1}$ gelatin) at 4°C or up to 3 months at room temperature without any significant loss of activity (Zlotkowska and Ilnicka-Olejniczak, 1993).

Starter culture activity is affected by the rate of cooling after incubation, level of acidity at the end of the incubation period and the temperature and duration of storage (see above the working or reserve stock culture). Cooling is important to control the metabolic activity of the starter. Goat's milk can also be used as a medium for the growth and maintenance of yogurt and lactococcal cultures (Abrahamsen *et al*., 1982), but strongly flavoured milks either reduced or inhibited the growth of some cultures after 10 transfers; excess fatty acids in the strong goat's milk may have proved inhibitory.

8.2.2 Dried starters

An alternative method for the preservation of yoghurt starter cultures is drying. The different drying methods used are:

- Vacuum drying
- Spray drying

} (old methods not used at present time)

- Freeze drying or lyophilisation (widely used in the laboratory)
- Freeze drying of concentrated cultures (widely used commercially).

The main objectives behind these developments are first, to reduce the workload which is involved in maintaining liquid cultures, second, to improve the shelf life of the preserved cultures, and third, to facilitate the dispatch of cultures by post without any appreciable loss in their activity.

According to Tofte-Jespersen (1974a,b, 1976), the drying process prior to 1950s was carried out under vacuum and the results were not encouraging (i.e. the preserved dried cultures contained only 1–2% viable bacteria). To regain maximum activity several subculturings were required. In essence, this method of preservation consisted of taking an active liquid starter culture, adding lactose as a protective agent and calcium carbonate to neutralise the excess acid, followed by partial concentration of the mixture (i.e. removal of whey). The concentrated starters, which were by then in a granular form, were dried under vacuum.

Owing to the poor results achieved by vacuum drying, alternative methods were sought, and one of these methods was spray drying which was first used in the Netherlands for the preservation of cheese starter cultures (Stadhouders *et al.*, 1969). Although the results proved promising, this technique has not been developed commercially. However, the Dutch process could be summarised as follows:

- Hydrolyse milk protein with trypsin for 4 hours at 37°C followed by steaming.
- Propagate the starter culture at 20°C with pH control using $Ca(OH)_2$ as a neutralising agent.
- Evaporate the starter at 27°C to 22 g 100 g^{-1} TS (total solids) and spray dry (air temperature 70°C) to 9 g 100 g^{-1} moisture with the powder temperature not exceeding 42°C.
- Vacuum dry at 27°C and 1–2 mm Hg; the dried culture has about 5 g 100 g^{-1} moisture.

Work in the United States (Porubcan and Sellars, 1975a) showed that the addition of certain compounds, for example ascorbic acid and monosodium glutamate, helped to protect the bacterial cells during the drying process. Furthermore, Porubcan and Sellars (1975a) recommended that starter cultures must be propagated in a buffered medium. The objectives of buffering are firstly, to increase the number of viable organisms/volume of sample and secondly, to neutralise certain metabolites, mainly lactic acid, which can inhibit bacterial growth beyond a certain level. Cultures preserved by this process retained their activity after storage for 6 months at 21°C.

Another method of spray drying yoghurt cultures was developed in Sweden (Anderson, 1975a, b) for which the advantages of drying at high temperatures (75–80°C) without causing any bacterial damage and maintaining different ratios of *S. thermophilus*:*L. delbrueckii* subsp. *bulgaricus* in the preserved culture were claimed. For example, a ratio of 40:60 in a dried culture can be used for the production of a sharp flavoured yoghurt (due to high level of *L. delbrueckii* subsp. *bulgaricus*), while for a milder flavoured yoghurt a ratio of 60:40 can be used. The Swedish method of spray drying can be summarised as follows:

- propagate the starter culture in sterilised concentrated skimmed milk (18–24 g 100 g^{-1} TS);
- fortify the growth medium with lysine, cystine and cyanocobalamine;
- dry at a temperature of 75–80°C.

Although this development claimed many advantages, the system is not widely used.

Recently, Teixeira *et al*. (1994, 1995a–c) reported that the death kinetics of *L. delbrueckii* subsp. *bulgaricus* using the spray drying process were influenced by many factors such as (a) to (f). (a) The logarithmic survival ratio decreased with increased outlet air temperature with first-order kinetics and the pseudo-z for the organism was about 17°C. (b) The calculated activation energy (E_a) values were 33.5 kJ mol^{-1} above 70°C and 86 kJ mol^{-1} at less than 70°C. (c) The relationship between the entropy and enthalpy of activation for spray drying and heating in liquid medium was linear; the data for drying, however, fell in the range of negative entropy. (d) High storage temperature and water activity reduced the survival of the dried cells. (e) The survival rate was higher in the presence of mono-Na-glutamate and ascorbic acid during storage at 4°C compared with 20°C. (f) The dried cells were sensitive to certain inhibitors (e.g. penicillin, pyronin Y, lysozyme and sodium chloride) due to damage of DNA, cell wall and cell membrane, respectively (see also Riis *et al*., 1995; Teixeira *et al*., 1996, 1997).

The addition of dextrin prior to drying, using a two-fluid atomiser and decreasing the air outlet temperature improved the survival rate of the yoghurt organisms (Metwally *et al*., 1989; Abd-El-Gawad *et al*., 1989). Other additives and/or methods of drying that improved the survival rate of yoghurt starter cultures and *L. acidophilus* may include the addition of betaine to an osmotically stressed medium protected some lactobacilli species, although not *L. delbrueckii* subsp. *bulgaricus* (Kets *et al*., 1996; Kets, 1997), drying the cultures on porcelain beads (Magdoub *et al*., 1987) or silica gel (de Silva *et al*., 1983), the use of whey supplemented with yeast extract plus glucose as a cryoprotective agent (Gandhi and Shahani, 1994) and drying the cells in a fluidised-bed dryer (Rossi and Clementi, 1987).

Freeze-dried or lyophilised yoghurt cultures are produced when the starter culture is dried in the frozen state. This method of starter preservation enjoys widespread popularity and aims to increase the reliability of the preserved cultures, that is, the dried cultures should provide a high number of viable organisms and the maximum percentage survival during storage, compared with vacuum or spray-dried starter cultures (see Cattaneo *et al*., 1986; Porubcan, 1990).

In lyophilised cultures, the survival rate is high and only a small quantity is needed to inoculate the mother culture. The number of viable bacteria/unit of addition is of the same order of magnitude as in the liquid starter culture (i.e. 2 ml 100 ml^{-1}) (Tofte-Jespersen, 1974b, 1976). It can be observed, however, that freezing and drying can damage the preserved organisms and, in particular, the bacterial cell membrane. Thus, Porubcan and Sellars (1975b) filed a patent in the mid 1970s for the production of freeze-dried starter cultures with the growth medium fortified with certain additives to minimise the damage to the bacterial cell membrane. The growth medium consisted of the following components:

- milk base (pH adjusted to 6.0–6.5),
- additives (e.g. ascorbic acid, mono-Na-glutamate, aspartate compound),
- cryoprotective agents (e.g. inositol, sorbitol, mannitol, glucose, sucrose corn syrup, dimethyl sulphoxide (DMSO), PVO, maltose, mono- or disaccharides).

Another approach used by Morichi (1972, 1974) to minimise bacterial cell damage was the addition of certain cryogenic agents to the cell suspension. The protective solutes are of a hydrogen bonding and/or ionising group in nature. Hence, these

compounds stabilise the cell membrane and so prevent, to a certain degree, cellular injury during preservation procedures. The effect of such solutes on the survival of the yoghurt organisms and *L. acidophilus* is illustrated in Table 8.1. It can be concluded from the work of Morichi (1972) that the survival of *L. delbrueckii* subsp. *bulgaricus* was enhanced by L-glutamic acid, L-arginine and acetyl glycine, and that of *S. thermophilus* and *L. acidophilus* by the above mentioned compounds and DL-pyrolidone carboxylic acid and DL-malic acid; furthermore, *L. delbrueckii* subsp. *bulgaricus* is more vulnerable to cellular damage than *S. thermophilus* (see Table 8.1).

Thus, due to the low survival rate of starter cultures, the early commercial freeze-dried cultures were not suitable for DVI and it was necessary to propagate these cultures a few times to re-establish their activity prior to fermentation (Porubcan and Sellars, 1979). In the 1980s, Amen and Cabau (1984, 1986) patented a process in which cheese starter cultures were grown in a special medium containing a nutritive substrate, and the pH was maintained >5.5 by the addition of a neutralising agent such as ammonium hydroxide. The removal of the inhibitory ammonium lactate was carried out by UF and the addition of water (see Section 8.3.2). The concentrated culture was then freeze dried and made suitable for DVI application; it is safe to assume that a similar approach could be used for concentrating yoghurt starter cultures for use in DVI systems.

In view of the relative susceptibility of the yoghurt organisms to the freeze-drying process, many different protective compounds have been studied and some examples are shown in Table 8.2. Milk solids are widely accepted as very good cryogenic agents for the preservation of starter cultures and the use of levels up to 20–25 g $100\,g^{-1}$ TS has been reported (see Table 8.2); however, 16 g $100\,g^{-1}$ TS in the growth medium is a realistic level and a typical procedure for the preservation of a mixed strain yoghurt starter culture was reported by Tamime and Robinson (1976).

It is evident that the survival rate of lyophilised starter cultures is influenced by several factors.

8.2.2.1 Growth medium

Skimmed milk and/or whey supplemented with yeast extract or hydrolysed protein are good growth and suspension media for the preservation of freeze-dried cultures (Alaeddinoglu *et al*. 1989). The organisms should be propagated at their optimum

Table 8.1 Effect of certain cyrogenic agents (adjusted to pH 7 and 0.06 M) on the survival rate of freeze-dried yoghurt bacteria[a]

Cryogenic agent	*S. thermophilus*	*L. delbrueckii* subsp. *bulgaricus*	*L. acidophilus*
L-Glutamic acid	35–40	16–21	42–63
L-Arginine	21–40	20–35	39–57
L-Lysine	6–7	1–10	4–38
DL-Threonine	7–11	6–10	6–21
Acetyl glycine	29–44	7–33	3–35
DL-Malic acid	52–59	6–15	28–66
DL-Pyrolidine-carboxylic acid	24–48	9–11	24–56

[a] Figures as percentage of original numbers; the range of survival is due to different strains tested. Adapted from Morichi (1972).

Table 8.2 Selection of different cryogenic compounds employed during the production of freeze-dried yoghurt starter cultures

Method of preparation	References
Skimmed milk + lactose or horse serum + glucose or Naylor and Smith (1946) reducing medium	Briggs *et al*. (1955)
Skimmed milk + peptonised milk + saccharose + Na-glutamate	Gavin (1968)
Suspend concentrated culture in sucrose solution and buffering salt + Na-glutamate	Bannkova and Lagoda (1970)
Suspend washed cells in skimmed milk + ascorbic acid + thiourea + ammonium chloride	Sinha *et al*. (1970, 1974)
Mix active culture + sucrose, gelatin and Na-glutamate + Na-citrate (for streptococci) or Na-acetate (for lactobacilli)	Lagoda and Bannikova (1974, 1975)
Suspend cell biomass in gelatin + Na-citrate + mono-Na-glutamate + sucrose or only malt extract + Tween 80 (see text)	Speckmann (1975) Porubcan and Sellars (1975b)
Suspend biomass in lactose or mono-Na-glutamate or arginine hydrochloride	Pettersson (1975a, b)
Grow culture in MRS broth or all purpose Tween + cryogenic agents[a]	Kilara *et al*. (1976)
Grow culture in low lactose medium + soya + casein and continuously buffer	Hup and Stadhouders (1977)
Mix culture with sugar solution or peptone or polymer 1500	Nikolova (1978)
Skimmed milk + yeast extract + Vit. E or Tween 80 + sheep's serum	Naghmoush *et al*. (1978)
Skimmed milk + Na-glutamate + sucrose	Ozlap and Ozlap (1979), Kim *et al*. (1988)
Suspend cell biomass in skimmed milk + sucrose + Na-glutamate + buffering salt	Lagoda *et al*. (1983)
Mix cell biomass with SMP + corn syrup or lactose	Ishibashi *et al*. (1985)
Mix cell biomass + sucrose + Na-citrate + gelatin + mono-Na-glutamate or skimmed milk + Na-glycerophosphate	Bozoglu *et al*. (1987)
Suspend culture in whey or skimmed milk + sucrose + adonitol + glycerol	Alaeddinoglu *et al*. (1989)
Suspend culture cells in skimmed milk + sucrose + Na-glutamate	Gupta and Ratnakar (1990)
Suspend cell biomass in skimed milk + glycerol + glucose + dimethyl sulphoxide + polyethelene glycol	Wolff *et al*. (1990)
Mix cell biomass with skimmed milk + whey base medium + Tween 80	Champagne *et al*. (1991a)
Mix cell biomass + milk + glucerol	Béal and Corrieu (1994)
Mix the cultures with sucrose + Mg-sulphate + ascorbic acid + Na-acetate + apilac[b] + Ca-hydroxide	Belov *et al*. (1995)

[a] Cryogenic agents for lactobacilli: casitone, lactose, malt extract, milk solids, mono-Na-glutamate, Myracet®, whey powder and/or peptonised milk; for streptococci: same as lactobacilli + dimethyl sulphoxide, glycerol and/or pectin. [b] Apilac® is a lyophilised preparation based on bee-collected pollen.

temperatures. However, improved survival rate during freezing and freeze drying of *L. delbrueckii* subsp. *bulgaricus* was achieved after growth in the presence of calcium (Wright and Klaenhammer, 1983). Also neutralisation of the growth medium to pH range 5–6 is recommended.

8.2.2.2 Cell biomass and suspension medium

Providing a culture $>10^{10}$ cfu ml^{-1} (see Section 8.3.2) including neutralisation of the suspension medium improves the survival rate of the starter culture in the presence of cryoprotective agents (see Table 8.2; Font de Valdez *et al*., 1983a, b, 1985a). Removal of carbonyl compounds from the growth medium is also recommended as they can react with amino groups in the bacterial cells and can accelerate their death (see the review by Champagne *et al*., 1991b). Growth of *L. delbrueckii* subsp. *bulgaricus* in a medium at constant pH 5.7 and fortified with Na-citrate and Tween 80 improved the survival rate by a factor of ten (Champagne *et al.*, 1991a). However, the addition of certain polymers (gelatin, xanthan gum and maltodextrins) had a detrimental effect of the stability of *S. thermophilus* during storage at 20°C, whilst α- and β-galactosidase activity losses in *Bifidobacterium longum* during storage at 20°C were greater than parallel cultures stored at 4°C or –20°C (Champagne *et al*., 1996).

8.2.2.3 Freeze-drying, packaging and storage

Wolff *et al.* (1990) reported that vacuum freeze drying was more suitable for *S. thermophilus* compared with atmospheric pressure freeze drying; suspension of the cells in reconstituted skimmed milk provided good protection for the cells. Whilst storage of the dried culture under vacuum or nitrogen provided better survival of the yoghurt cultures, *S. thermophilus* was found to preserve well, while *L. delbrueckii* subsp. *bulgaricus* was more sensitive to freezing and drying (Bozoglu *et al*., 1987). In recent studies reported by Castro *et al*. (1995, 1996, 1997), the survival of lactobacilli was greatest when the dried culture was stored at 11% relative humidity and 5°C.

8.2.2.4 Reactivation

It is recommended that users follow the instructions of the starter culture manufacturer. However, rehydration medium and temperature (i.e. 20°C) can affect the leakage of cellular ribonucleotides from damaged cells. Detailed studies on both mespohilic and thermophilic lactic acid bacteria have been reported by Morichi *et al*. (1967) and Font de Valdez *et al*. (1985b–e, 1986) (see also the review by Tamime, 1990).

Lyophilised cultures tend to have a long lag phase and need to be subcultured at least twice to obtain an active liquid culture. Hence, for the production of bulk starter, System 1 is used (see Fig. 8.1), otherwise large quantities of dried culture are needed for direct inoculation of the bulk starter and a long incubation period is required. This approach is not advisable for two main reasons, first, the bulk starter may not be active when used for the manufacture of yoghurt and second, from an economic point of view, the approach can be very costly. More recently, concentrated freeze-dried cultures have appeared on the market and it is feasible to use such cultures for direction inoculation of bulk starter (see Fig. 8.1, System 2) or alternatively, for DVI of milk for the manufacture of yoghurt (see Fig. 8.1, System 3; Gatto *et al*., 1993; Kreuder *et al*., 1994; Riis *et al*., 1995). In both cases, although the

production time may be extended by 2–3 hour, considerable savings can be achieved by eliminating the need for trained personnel to handle the starter cultures.

8.2.3 Frozen starters

Yoghurt starter cultures can be also be preserved in the frozen form and such cultures are produced by two different routes:

- Deep or subzero freezing (–30 to –80°C)
- Ultra low temperature freezing (–196°C) in liquid nitrogen

Sterile liquid milk freshly inoculated with an active starter culture is deep frozen at –30 to –40°C to preserve the mother or feeder culture. Such frozen cultures can retain their activity for several months when stored at –40°C and this method of culture preservation became popular in the dairy industry because deep frozen cultures produced in centralised laboratories could be dispatched to a dairy in dry ice whenever required. These cultures are mainly packed in plastic containers and a typical example is the Astell-type plastic bottle (see Fig. 8.6). The reactivation procedure for these deep frozen cultures is as follows:

- Remove starter from freezer, i.e. at –40°C,
- Thaw the starter very quickly in water bath at 20°C,
- Incubate at 42°C until the desired acidity is reached,
- Cool and store overnight in the refrigerator,
- Subculture for the propagation of feeder for bulk starter (see Fig. 8.1, System 1).

An alternative type of deep frozen culture involves freezing an active liquid starter at –40°C. The process consists of propagating the culture in a continuously neutralised growth medium in order to optimise the bacterial cell number per millilitre. The bacterial mass is then separated using a Sharples separator (see Section 8.3.2) and the cells are resuspended in a sterile growth medium and/or protective agent prior to packaging and freezing. As mentioned earlier, the preserved cultures must be stored at –30°C to –40°C and be dispatched to dairies in insulated boxes filled with dry ice.

The freezing process can cause damage to the bacterial cells in particular to *L. delbrueckii* subsp. *bulgaricus*, and hence the activity of deep frozen cultures may tend to deteriorate after a certain time of storage due to several factors.

8.2.3.1 Growth medium plus cryogenic compounds

Imai and Kato (1975) reported that an improved medium for frozen cultures at –30°C contains skimmed milk supplemented with sucrose, fresh cream and $CaCl_2$ or gelatin. The same workers also observed that the presence of sodium acetate caused the starters to become sensitive to injury. In addition, concentrated cells (10^{10}–10^{12} cfu ml^{-1}) frozen at –30°C in the presence of certain mixtures of cryogenic compounds (Na-citrate, glycerol, Na-β-glycerophosphate, yeast extract, calcium sucrose, cream, sterile skimmed milk, peptone or lactose) have retained the activity of dairy starter cultures (Wright and Klaenhammer, 1983; Fayed *et al.*, 1985; Abraham *et al.*, 1990; Tamime, 1990; see also de Antoni *et al.*, 1989; Zlotkowska *et al.*, 1993). Other factors which affect the survival rate of *S. thermophilus* during freezing are growth phase and strain variation (Morice *et al.*, 1992); the

factors affecting the survival of lactobacilli have been reviewed by de Antoni *et al.* (1989).

8.2.3.2 Methods of concentration
Refer to Section 8.3.2.

8.2.3.3 Temperature of freezing and storage
Although freezing and storage at –40°C has proved to be a successful process for preserving dairy starter cultures, storage at –80°C to –100°C in liquid nitrogen vapour improves the survival rate of the organisms; also the rate of freezing should not be overlooked (Tsvetkov and Shishkova, 1982; Kim and Yu, 1990; Foschino *et al.*, 1992; Béal *et al.*, 1994).

8.2.3.4 Effect of thawing
Freezing and thawing can damage the cell membrane of *L. delbrueckii* subsp. *bulgaricus* and can induce sensitivity to NaCl and liver extract. The amino acid transport system of cells can also be damaged, but such cell injury is reversible if the cells are suspended in a solution of pyruvate, KH_2PO_4 and $MgSO_4$ (Font de Valdez *et al.*, 1993; Libudzisz and Mokrosinska, 1995; Oberman *et al.*, 1995; Piatkiewicz and Mokrosinska, 1995).

8.2.3.5 Miscellaneous factors
The destruction of bacterial cells during freezing is mainly due to an increased concentration of electrolytes and other solutes both inside the cell and in the suspending medium, rather than to mechanical damage as the result of ice crystal formation (Keogh, 1970). The former situation results in the denaturation of protein components and enzymes of the bacterial cell, while the concentration of electrolytes outside the cell results in the dehydration of the protoplasm due to the diffusion of water through the cell wall membrane. The kinetics of freezing and thawing processes of concentrated cell biomasses of lactic acid bacteria have been reported by Walczak *et al.* (1995).

Ultra low temperature freezing at –196°C in liquid nitrogen is by far the most successful method of preserving starter cultures. The reviews by Gilliland and Speck (1974) and Hurst (1977) illustrate the earlier research work which has been carried out in this field. The advantages of this technique of starter preservation have been summarised by Keogh (1970): at such low temperatures, the water molecules do not form large size crystals and the biochemical processes inside the cells cease to function, so that in biological terms, the bacterial cell is at a standstill.

Based on published reviews carried out in this field, the freezing and thawing cycle is still regarded as the most important factor in the successful use of frozen cultures in the dairy industry. An organism which is highly susceptible to damage during freezing is *L. delbrueckii* subsp. *bulgaricus*, but it was found that the presence of Tween 80 and Na-oleate improved cell stability (Smittle *et al.*, 1972, 1974; Smittle, 1973). *L. acidophilus* is also susceptible to freezing and thawing, and the injury is associated with cell wall components other than peptidoglycan; such injury is reversible by natural repair of the cell wall components (Johnson *et al.*, 1984). However, the type of growth medium, neutraliser used and/or cryoprotective compound(s) can play a major role in the activity of the preserved culture and the reviews by Gilliland (1977, 1985) highlight these factors in relation to different

species of lactic acid bacteria. However, Bulgarian workers reported that good results were obtained when yoghurt starter cultures were frozen at 0.36 ml 100 ml^{-1} of lactic acid (Tamime and Robinson, 1976; Tamime, 1990).

Mitchell and Gilliland (1983) managed to grow *L. acidophilus* in a medium of 2.5 ml 100 ml^{-1} pepsinised whey, maintained at pH 6.0 using a neutraliser consisting of sodium carbonate in ammonium hydroxide. The cell count was about 10^9 cfu ml^{-1} and after freezing in liquid nitrogen, the stability of the culture was excellent during storage for 28 days.

In order to maintain the activity of the preserved starter at ultra low temperatures, the cultures are neutralised, concentrated, packaged and finally frozen. The various stages in the production of such cultures have been reported by Tofte-Jespersen (1974b) and Porubcan and Sellars (1979). Normally the concentrated starter is packed in an aluminium can of 70 ml capacity (i.e. the recommended volume to inoculate 1000 l of milk). The cans are fitted with a pull-ring type closure, which is convenient for easy opening. However, for smaller quantities (e.g. 5 ml) the culture can be packed in a screw-capped polypropylene ampoule which resists cracking in liquid nitrogen. Another type of packaging material which may be used is the laminated carton. While the aluminium can and the polypropylene ampoule are stored in liquid nitrogen, the laminated carton is stored in a special container in an atmosphere of nitrogen vapour. Incidentally, the latter type of packaging material is used to pack pelleted concentrated frozen starter cultures (see Fig. 8.2).

Fig. 8.2 Preservation and packaging systems used for stock and DVI starter cultures

A, Liquid (incubate and store under refrigeration); B, litmus milk (partially incubated followed by refrigeration); C, D, E, frozen at –30°C (inoculate, incubate and then freeze, or inoculate and freeze); F, concentrated low temperature frozen cultures (starter in granular form); G, frozen in liquid nitrogen (polyproplyene ampoule); H, frozen in liquid nitrogen (pull-ring can); I, freeze dried; J, concentrated freeze dried.

These developments in the liquid nitrogen freezing of yoghurt starter cultures are primarily aimed at preserving the feeder/intermediate culture for the preparation of bulk starter (see Fig. 8.1, System 2). However, the ultimate objective is to employ such cultures for DVI of milk for the production of yoghurt (see Fig. 8.1, System 3), for although their use can lead to a slightly prolonged manufacturing time (longer lag phase – see later), the advantages can be summarised as follows:

- convenience
- culture reliability
- improved daily performance
- improved strain balance
- greater flexibility
- better control of bacteriophages
- improvement in quality of product.

In practice, certain drawbacks may be encountered, such as: (a) too great a dependence of the dairy on the starter manufacturer, (b) non-availability of liquid nitrogen facilities; at present, special containers are supplied by the starter manufacturer to customers for the transportation and storage of cultures in liquid nitrogen, (c) apportioning of responsibility in case of starter failure, (d) a natural reluctance within the dairy industry to introduce new technology in place of one that is well established as satisfactory, and (e) the longer time required for manufacture of yoghurt.

It is of the utmost importance that the thawing and handling of frozen cultures is carried out according to the supplier's recommendations (see also Tamime, 1990). A typical procedure is as follows:

- Remove can from liquid nitrogen storage.
- Thaw in water containing 100–200 $\mu g\, g^{-1}$ hypochlorite solution at 20°C for 10 min.
- When culture is partially thawed (i.e. contents are just loosened), remove can from water, open lid and add directly to bulk starter milk or milk for processing.

Yoghurt starter cultures are available in a number of forms (see Fig. 8.2) and depending on the method of preservation, the viable cell counts can vary slightly; Table 8.3 illustrates some typical differences and the blends of organism between the available types of commercially produced yoghurt and related starter cultures.

8.3 Technology of cell biomass production

It can be observed from the information on starter culture preservation that the survival rate is dependent on the processing conditions (growth medium, presence of cryogenic compounds, freezing and drying) and on the method of harvesting the cells. One of the main criteria of success during the preparation of the starter is the production of an active culture, that is a starter which consists of very large number of viable cells, so that when it is added to milk the fermentation process is initiated as quickly as possible.

Table 8.3 Enumeration of lactic acid bacteria in commercial DVI starter cultures (cfu g^{-1}) used for the manufacture of bio fermented milks and yoghurt

Starter culture	*Bifidobacterim* spp.[a]	*L. acidophilus*	*S. thermophilus*	*L. delbrueckii* subsp. *bulgaricus*
Freeze dried	4.6×10^{11}	1.4×10^{11}		
	1.1×10^{10}	5.6×10^{9}		
	4.8×10^{9}	7.1×10^{9}	7.5×10^{9}	
	3.6×10^{9}	1.2×10^{10}	4.2×10^{10}	
	5.4×10^{9}	2.1×10^{10}	1.7×10^{11}	
	3.4×10^{8}	4.8×10^{8}	8.7×10^{7}	
			6.6×10^{10}	2.5×10^{8}
Frozen	4.6×10^{7}	6.9×10^{9}	4.3×10^{7}	

[a] *B. bifidum*, *lactis*, *longum* and/or *infantis*.

Data compiled from La Torre (1997).

8.3.1 Growth characteristics

During the growth of any dairy starter culture, the cells divide and increase in number up to a certain level and then start to die. This behaviour gives rise to the characteristic "growth curve" illustrated in Fig. 8.3, where it can be seen that the rate of cell division is divided into four different sections:

- Lag phase – this is the phase which follows immediately after inoculation of the milk. The delayed bacterial activity could be due to adjustment or adaptation of the organism to a new medium.
- Log phase – during this phase the cells display maximum activity, i.e. shortest generation time, as long as optimum conditions (nutrients and temperature) are available.
- Stationary phase – at a certain point, the cell viable number remains constant due to a lack of nutrients and an accumulation of waste metabolites (e.g. lactic acid in milk); the death of old cells and the production of new cells is in balance.
- Death phase – the number of viable cells starts to diminish, mainly due to unfavourable growth conditions.

It is safe to assume that there is a direct relationship between the activity of the starter and its age, and that an active culture falls somewhere on the growth curve between the upper middle region of the log phase and the beginning of the stationary phase (see Fig. 8.3). Therefore, the most active type of starter is the liquid culture, which is characterised by having a short lag period followed by a rapid rate of acid development; on average the inoculation rate may vary between 2 and 3 ml 100 ml^{-1} and the starter may contain in excess of 10^8 cfu ml^{-1} or g^{-1}. Nevertheless, if such a culture is used for preservation, undoubtedly the survival rate will be low and may not be suitable for DVI of the milk. Therefore, cell biomass production becomes an important criterion in culture preservation.

8.3.2 Concentration of the cell biomass

Batch and continuous fermentation are used for the production of dairy starter cultures. The fermentation kinetics of *L. delbrueckii* subsp. *bulgaricus* have been

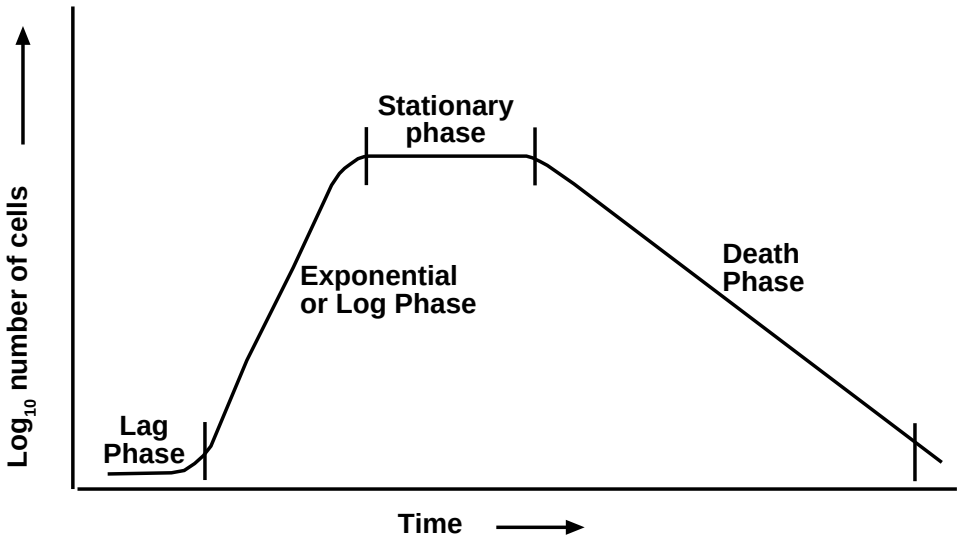

Fig. 8.3 Growth curve characteristics of a population of viable unicellular micro-organisms

examined by Venkatesh *et al*. (1993) who concluded that the relationship between adenosine biphosphate (ATP) concentration and cell biomass in a batch fermentation could be approximated by a Leudeking–Piret relationship and that cell productivity using continuous fermentation was three times higher than using a batch process. Thus, the systems which are used for the concentration of cell biomass are as follows.

8.3.2.1 Mechanical means

The equipment most widely available in the dairy industry (e.g. Sharples separator, desludging separator, clarifiers or bactofuge) may cause some physical damage to the bacterial cells, thus reducing the rate of survival during the preservation stage. Alternatively, ultracentrifuges (20 000 *g*) can be used and it is possible that these cause least physical damage to the cells. Béal and Corrieu (1994) concentrated yoghurt cultures (i.e. single and mixed strains) by centrifugation at 11 000 *g* for 15 min at 4°C at the end of the log phase following growth in a batch system with the pH controlled by adding 10 mol l^{-1} of NaOH. The cultures were either frozen at –75°C or freeze dried. They concluded that: (a) during concentration and preservation, culture activity was not altered significantly, (b) during storage for 24 weeks, the viability of the cultures decreased continuously and the decrease was greater in mixed cultures, and (c) the frozen cultures showed greater resistance during storage than the freeze-dried type (see also Lelieveld, 1984; Béal *et al*., 1989).

Shear stress during culture growth of *L. delbrueckii* subsp. *bulgaricus* affected the characteristics of the organism, for example, cell elongation, membrane permeability and resistance to freezing at –80°C. Arnaud *et al*. (1993) observed that, at a shear

stress level of 36 Pa, biomass concentration was higher and the lag time shorter compared to the same culture grown at 72 Pa.

8.3.2.2 Chemical neutralisation

The two different organisms in the yoghurt starter cultures can tolerate different levels of acidity in the growth medium, with *S. thermophilus* being more sensitive to lactic acid than *L. delbrueckii* subsp. *bulgaricus*. Thus, while lactobacilli can survive beyond 2 g 100 g^{-1} lactic acid, the streptococci can tolerate up to 1.2–1.5 g 100 g^{-1} lactic acid, and hence it is essential that the lactic acid is either removed or neutralised in order to protect *S. thermophilus* (see also Benthin and Villadsen, 1995). Sodium or ammonium hydroxide is widely used, but the latter compound is usually recommended. The reaction between lactic acid and the neutralising compounds results in the formation of sodium or ammonium lactate. However, at a certain level, lactate starts to inhibit the starter organisms also, and as a result the cell biomass concentration is limited to 10^{10} cfu ml^{-1} or g^{-1}. Therefore, to achieve a high concentration of about 10^{11}–10^{12} cfu g^{-1}, for example, in a freeze-dried starter culture, the cell biomass grown in a chemically neutralised system requires further concentration, possibly using a mechanical separator (see also Amen and Cabau, 1984; Barach and Kamara, 1986; Parente and Zottola, 1991; Borzani *et al*., 1993).

Since the 1980s, there have been great technological developments by starter culture manufacturers in the production and preservation of DVI freeze-dried and frozen cultures. Martin (1983) described the production of the freeze-dried type by Rhodia Texel in France (formerly known as Eurozyme). An illustration of a fermentor used to produce high numbers of cells is shown in Fig. 8.4.

8.3.2.3 Diffusion culture

This technique involves the use of selected semipermeable membranes to concentrate micro-organisms and, in principle, this process consists of the following steps: (a) growth of the starter culture in a restricted volume of medium, (b) provision of a system that allows fresh growth nutrients to permeate in through the membrane, and (c) allows the metabolic waste materials to diffuse out.

This constant replenishment of the medium allows the concentration of bacterial cells to build up beyond normal levels and, using the diffusion culture technique with cheese starters, Osborne (1977) and Osborne and Brown (1980) have reported achieving $>10^{11}$ cfu ml^{-1}. Although the waste metabolites diffuse out from the growth medium, some of the lactate is retained and this does tend to limit the cell biomass concentration. No work has been reported on yoghurt organisms, but it is possible that the principle of this technique could be applied to concentrate *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*.

An alternative diffusion method used to concentrate starter cultures uses UF and electrodialysis. Boyaval *et al*. (1987, 1988) used the continuous fermentation of sweet whey permeate to produce lactic acid and cell biomass in a membrane bioreactor. The organisms were a mixed culture of *L. helveticus* and *S. thermophilus*. Steiber and Gerhardt (1980) used dialysis to concentrate *L. delbrueckii* subsp. *bulgaricus* in a continuous fermentor using deproteinised whey; the cell biomass was more than double that from an ordinary fermentor. A UF method was also used to concentrate *S. thermophilus* with cellular productivity nine times higher than that obtained by conventional methods (Prigent *et al*., 1988).

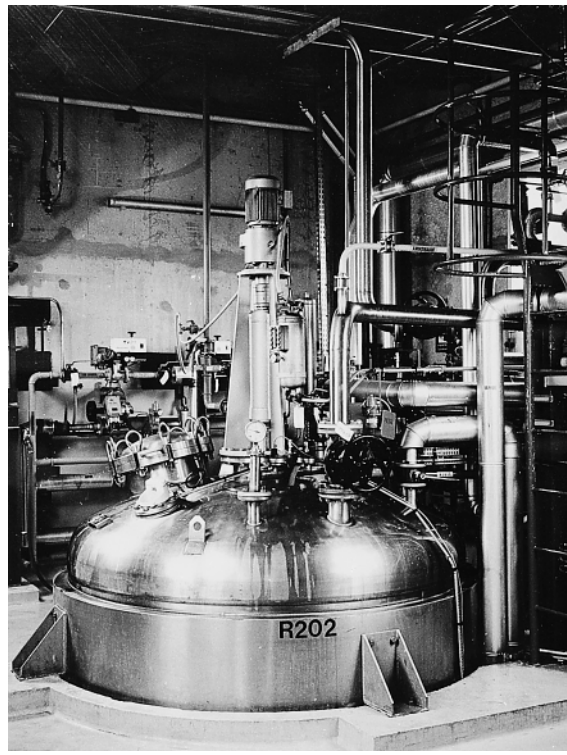

Fig. 8.4 Fermentor for the production of starter culture concentrate prior to freeze drying

Note: On-site view of a fermentation tank for the production of starter culture; ammonium hydroxide is used to neutralise the acid produced by the lactic acid bacteria.

Reproduced by courtesy of Rhodia Texel Ltd., Stockport, U.K.

8.3.2.4 Cell immobilisation in gels

This method of cell biomass concentration involves culture immobilisation in gum gels or porous foam-glass beads and this technique has reached commercial scale production. The published data available on the yoghurt cultures have been reported by Audet *et al.* (1989, 1990, 1991a, b), Buyukgungor and Caglar (1990), Buyukgungor (1992), Champagne *et al.* (1993), Ragout *et al.* (1996) and Turkur and Hamamci (1998). In essence, this technique involves entrapping the cell biomass in small beads (0.5–1.0 mm diameter) of κ-carrageenan/locust bean gum or Ca-alginate to give a concentration of around 10^9 cfu g^{-1}. The material can then be used for the continuous fermentation of milk, with the breakdown of lactose being achieved both by cells held in the solid matrix and cells released into the milk. As cells within the matrix are actively growing, such bioreactors can operate for long periods with selected, single cultures but, for yoghurt, maintaining the balance of cocci : rods could prove more difficult.

It has been suggested also that if the beads could be suspended in a physiologically neutral medium and perhaps deep frozen, this approach could provide an alternative means of preservation, with the supporting matrix acting as a protective agent. However, at the present time, the technology appears to be used for experimental purposes only.

8.4 Production systems for starter cultures

8.4.1 Introductory remarks

It is evident from the above information that the preserved cultures are relatively lower in activity compared with liquid culture. As a consequence, DVI starters (e.g. concentrated freeze-dried or frozen cultures) tend to show slightly longer lag phases.

Although the cell concentration is in the region of 10^9–10^{12} cfu ml^{-1}, the inoculation rate is relatively small. The use of higher inoculation rates is not recommended for two main reasons. First, it increases cost of production and second, it leads to excessive metabolic activity by the starter which can mean difficulties in controlling the fermentation process and the yoghurt can be of an inferior quality (i.e. bitter). In addition, the larger the inoculum of the starter culture (including liquid cultures), the greater the tendency for whey syneresis to occur in the retail yoghurt. Furthermore, the longer lag phase needed by these cultures is an indication that their metabolism at the time of inoculation is at a very low level, and hence more time is required for the essential adaptation. Incidentally the quality of the milk must be very good, because the presence of any inhibitory agents (e.g. antibiotics or detergent residues) can ultimately reduce the activity of the starter culture.

Currently, yoghurt starter cultures constitute mixed strains of different microorganisms. According to Stenby (1998) some of the criteria used to select strains for starter culture blends are:

- Acidity: Mild to medium or sharp taste in end product
  Post-acidification during storage (i.e. ability of strains to produce acid at low temperatures)
  Level of acetic acid (i.e. only for bio cultures)
- Flavour: Low, medium or high content of acetaldehyde
- Viscosity: Low, medium, high or very high
- Fermentation: Short (about 6 hours) or long (up to 16 hours) incubation
- Bacteriophages: Blend of bacteriophage unrelated strains

In some countries the statutory regulations may stipulate that there be a ratio of 1:1 between *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, a minimum number of cfu ml^{-1} in the final product and a pH level <4.4. Such constraints will limit the options of yoghurt manufacturers to diversify and provide consumers with a wide range of products. In some countries for instance, *L. delbrueckii* subsp. *lactis* and *L. helveticus* are blended with yoghurt organisms or in bio cultures other lactobacilli species and *Bifidobacterim* spp. are used. Thus, in order to maintain the starter culture characteristics mentioned above and to maintain the desirable counts of probiotic organisms (e.g. *L. acidophilus, L. paracasei* subsp. *paracasei*, *Lactobacillus rhamnosus* and/or *Bifidobacterium* spp.), the use of DVI starter added directly into the milk base (see Fig. 8.1, System 3) has become a popular practice in the industry. However, there is still a demand, especially in large factories, for traditional mixed starter cultures consisting only of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* for the manufacture of yoghurt.

Therefore, as illustrated earlier in Fig. 8.1, there are two main methods for the production of bulk starters. The first method (System 1) is a simple scale-up system

of starter propagation (stock → mother → feeder or intermediate → bulk); however, the second method (System 2) is DVI inoculation of the bulk starter. In either system the aim is to produce a pure active culture free from contaminants, mainly bacteriophages, and the different methods which have been devised may be divided into three main categories, simple microbiological techniques, employment of mechanically protected equipment and tanks and propagation of starters in bacteriophage resistant/inhibitory medium (BRM/BIM).

8.4.2 Simple microbiological techniques

In this system the equipment/materials are basically laboratory utensils and a starter tank and may consist of glass test tubes, McCartney bottles, 250 ml flasks (for propagation of mother culture), 2–5 l flasks (for production of feeder culture) and graduated and Pasteur pipettes.

Reconstituted skimmed milk powder (10–12 g 100 g^{-1} TS) is used as the growth medium and the glassware containing the milk is plugged with non-absorbent cotton wool. The whole is sterilised in an autoclave at 121°C for 10 min for small volumes (up to 250 ml) or for 15 min for larger quantities (2–3 l). However, the milk for the feeder culture is normally only steamed for 1 hour. It is recommended that a sample of sterilised milk for the mother cultures should be incubated at 30°C or 50°C for 2 days prior to use in order to check its sterility. Pipettes are sterilised in an oven at 160°C for a minimum of 2 hours.

The reactivation and subculturing procedures must be carried out under extremely hygienic conditions. For example, the freeze-drying ampoule is wiped with alcohol before breaking the glass, or alternatively, if a liquid stock culture is used, the lip of the test tube or McCartney bottle must be "flamed" over a bunsen burner when the cotton wool or the screw cap is removed, and again immediately before replacing it. It is also recommended that the starter working area and atmosphere must be clean (i.e. the air must be filtered) and, if possible, the whole starter laboratory should be under positive pressure so that unfiltered air does not enter the room whenever the door is opened. Alternatively, subculturing can be carried out under a laminar-flow hood to reduce the possibility of airborne contamination.

The production of a bulk starter using this system requires a simple tank design (i.e. batch pasteuriser/starter incubator). The tank is not pressurised and at the point of subculturing the lid is opened and the starter is poured into the milk. Very small quantities (45 l) of bulk starter can be produced in an ordinary milk churn or similar stainless steel container. A water bath or thermostatically controlled cabinet may be used as a combined pasteuriser, cooler and incubator for the production of limited volumes of bulk starter.

It is worthwhile mentioning at this stage that this method can also be used for the production of the feeder culture used for inoculating the mechanically protected Jones tank (refer to the review of Tamime, 1990).

8.4.3 Mechanically protected systems

Two aspects of starter production in mechanically protected systems are important: first, the growth medium is heat treated and cooled to incubation temperature in a completely enclosed vat and second, the inoculation of the starter takes place through a barrier which prevents the entry of air. Since 1950 there has been a great

improvement in starter culture equipment, mainly due to the centralisation of fermented dairy products manufacture and hence the demand for large quantities of bulk starter. As a result, different types of mechanically protected system have been developed; however, since the publication of the first edition of this book, few technical developments have occurred with respect to the design and structure of these tanks. The topic has been extensively reviewed elsewhere (see below for further information). As a consequence and in view of the wider application of DVI of milk for manufacture of yoghurt, only the main systems are described. Some examples of mechanically protected systems are given below.

8.4.3.1 The Lewis system

The development of this technique is well documented by Lewis (1956, 1987) and Cox and Lewis (1972) and involves the use of two-way hypodermic needles to carry out the transfer of stock to mother culture, mother culture to feeder culture to bulk starter; all inoculations take place through a barrier of chlorinated water. In order to facilitate easy transfer of the cultures during each stage of propagation, re-usable, collapsible polythene bottles are used (115 ml and 850 ml capacity) for the mother and feeder cultures, respectively. The polythene bottles are fitted with Astell rubber seals and a screw cap. These bottles are filled with the growth medium (10–12 g 100 g^{-1} reconstituted skimmed milk free from antiobiotics), sealed and capped; the contents are thus isolated from aerial contamination throughout the sterilisation, inoculation and incubation stages. At the point of intermediate transfers, the annular space of the Astell rubber seal is flooded with 100–200 mg l^{-1} hypochlorite solution, and finally the bottle containing the established culture is squeezed to discharge the inoculum. The overall technique is illustrated schematically in Fig. 8.5.

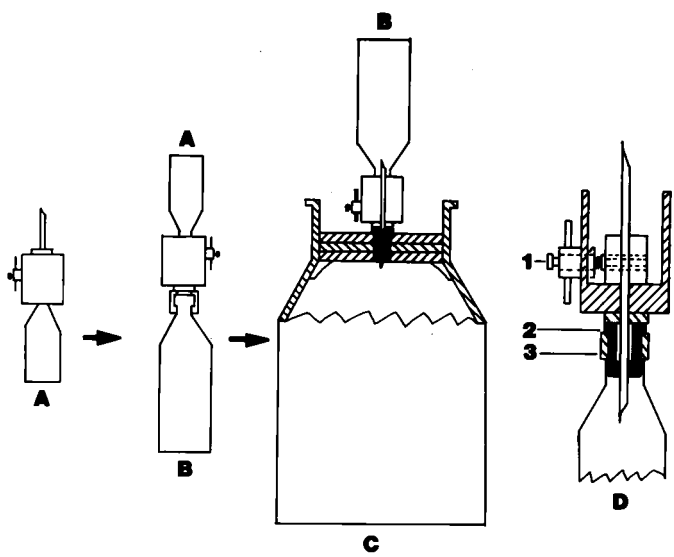

Fig. 8.5 Schematic illustration of the Lewis system for culture transfer

A, Mother culture; B, feed/intermediate culture; C, bulk culture; D, needle assembly (1, tap; 2, Astell seal; 3, hypochlorite solution).

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Fig. 8.6 On-site view of the Lewis system for culture transfer through a sterile water barrier

For the Lewis system, the milk is heated in a tightly sealed vessel and for safety reasons the tank is fitted with a pressure relief valve. During the heating stage some air may escape, but when the milk is cooled, no air enters the tank. The stainless steel pressure vessel is totally submerged within an insulated water tank, which provides maximum protection from aerial contamination as well as maintaining a constant temperature during incubation. The agitator shaft is fitted with a double mechanical seal, and water under pressure is fed to the seal housing to ensure efficient protection against contamination, cooling and lubrication. The transfer of the feeder culture to the bulk tank is carried out through a sterile barrier (i.e. water containing sodium hypochlorite solution). Figure 8.6 illustrates an on-site view.

8.4.3.2 The Jones system

The Jones tank is not a pressurised starter culture vessel, since air in the head space of the tank is forced out during heat treatment of the milk and enters again during the cooling stages. However, a slight positive pressure inside the tank can be achieved by incorporating a fan unit in the air filtration/sterilisation system. Detailed specifications of this bulk starter system have been reported by Tamime (1990) including a combined Lewis/Jones system.

8.4.3.3 Sterile and filtered air systems

Different types of bulk starter tank using filtered, sterile air (under positive pressure) have been made available to dairy processors in many countries by the major dairy equipment suppliers. One typical example using high efficiency particulate air (HEPA) filtration systems on bulk starter tanks was studied at NIZO (in the Nether-

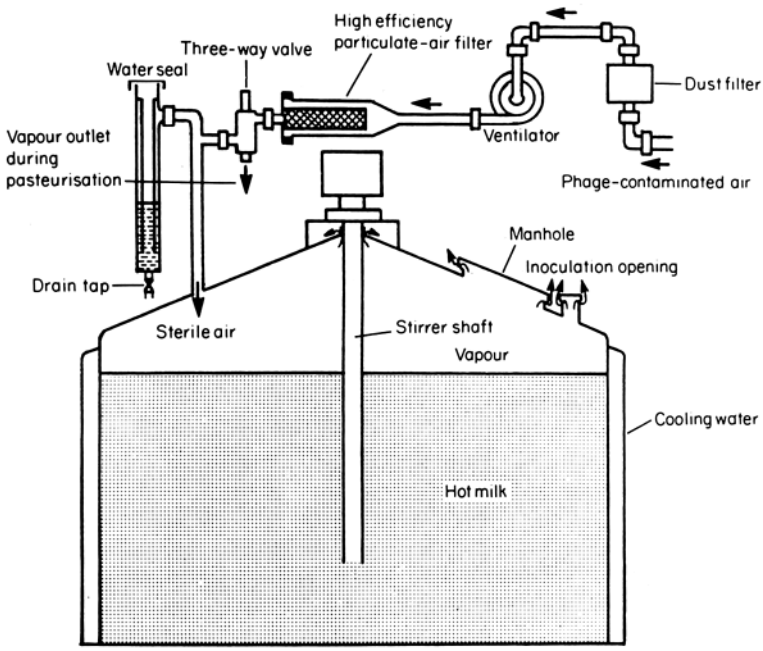

Fig. 8.7 Cultivation tank for production of bulk starter with over-pressure of sterile air

After Stadhouders *et al.* (1976) and Stadhouders (1986). Reproduced by permission of *North European Dairy Journal* and *Netherlands Milk and Dairy Journal.*

lands) in the 1970s and an illustration of the tanks is shown in Fig. 8.7 (see also Stadhouders *et al.*, 1976; Tofte-Jesperson, 1979; Stadhouders, 1986). Leenders *et al.* (1984) evaluated the effect of the HEPA filter [ultrapolymembrane PF-PP 30/3 (0.2 μm HF)] on air entering bulk starter tanks in factories and observed that less than one phage out of 1.9×10^8 phage passed through – this is a priority requirement.

8.4.3.4 The Tetra Pak system

This system is described in detail by Baudet (1983) and Bylund (1995) and in principle is somewhat similar to the Lewis method except in two respects. First, the tank is of a different design and is fitted with a special filter consisting of hydrophobic paper with prefilters on each side; the whole filter unit is enclosed in a protective casing. During the heat treatment of the milk, the air diffuses out through the filter from the tank, and vice versa during the cooling stages. It is critical that the filter sterilises the air to reduce the effect of airborne contamination. Second, in the Lewis system, starter transfer from one container to another relies entirely on squeezing the collapsible polythene bottle to eject the culture, while the Tetra Pak method uses sterilised air (Fig. 8.8).

Glass bottles are used for the propagation of the mother culture and stainless steel cannisters for the feeder/intermediate stage (see Fig. 8.9). The bottles are sealed with rubber stoppers and a metal screw cap with an annular space. During culture transfer two disposable sterile syringes are used. The first syringe, which is short, is connected to the air supply and is fitted with an aseptic filter to sterilise the air. The second syringe is long enough to reach the bottom of the glass bottle and

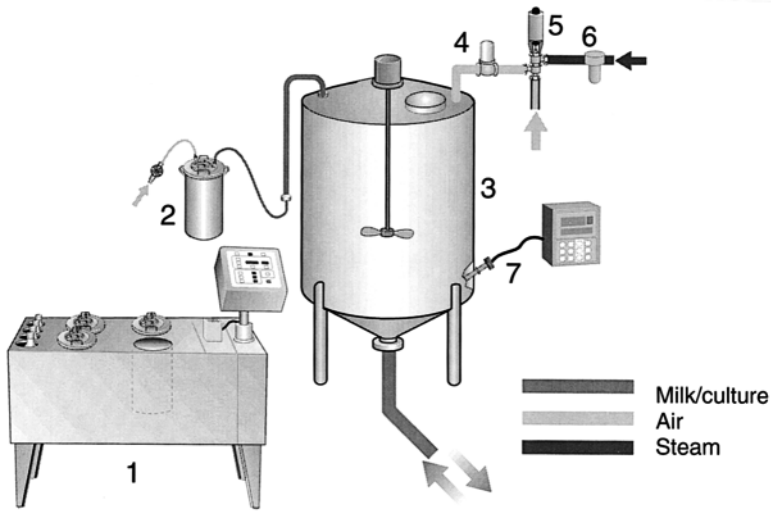

Fig. 8.8 Starter culture production using an aseptic transfer system

1, Incubator known as Viscubator; 2, feeder/intermediate culture container; 3, bulk starter tank; 4, HEPA filter; 5, air valve; 6, steam filter; 7, pH measurement unit.

Reproduced by courtesy of Tetra Pak (Processing Systems Division) A/B, Lund, Sweden.

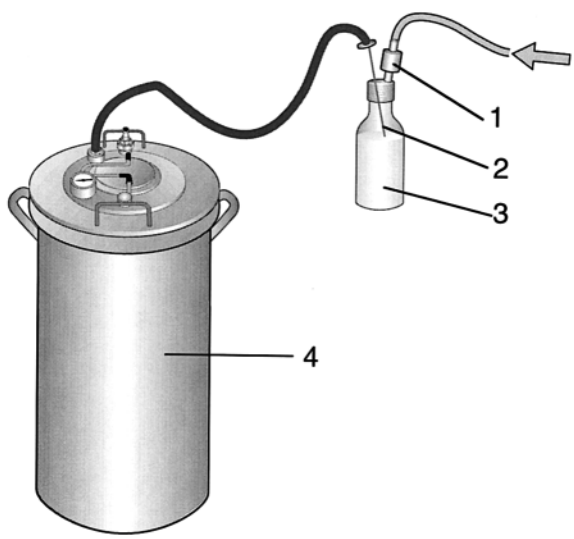

Fig. 8.9 Aseptic transfer of mother culture to feeder/intermediate culture container

1, Sterile filter; 2, aseptic needle; 3, mother culture glass bottle; 4, stainless steel feeder/intermediate container.

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is connected to the feeder container. Thus, when the supply is switched on, air is sterilised through the filter and enters the bottle via the short needle. This results in a buildup of pressure in the head space of the bottle, forcing the culture through the long needle into the feeder cannister. Incidentally, the bottles containing the skimmed milk are normally autoclaved and then cooled to the appropriate incubation temperature. The stock culture is injected into the bottle of the mother culture using a sterilised syringe inserted through the membrane or, alternatively, the freeze-dried stock culture is added into the bottle under aseptic conditions (i.e. the bottle cap is unscrewed in a laminar flow cabinet and the dried culture is added; another approach is to rehydrate the culture in sterilised milk and inject it into the bottle using a sterile syringe).

The feeder/intermediate culture is prepared using specially designed stainless steel containers as follows:

- Fill containers with skimmed milk and secure closure of the lid.
- Heat to 95°C for 30–45 min and cool to incubation temperature using the Viscubator (see Fig. 8.8).
- Transfer mother culture as described above, cool to <10°C; the culture is then ready to inoculate the bulk starter tank.

The feeder/intermediate containers have two special fittings, one for compressed air, and the other in the form of a tube made of stainless steel pipe which connects to the bulk starter tank during culture transfer. These fitments are equipped with special valves with quick release couplings. An on-site illustration of the feeder/intermediate culture container showing the pipe connections is shown in Fig. 8.10. A similar system of bulk starter production was reported by Rasic and Kurmann (1978) and an overall schematic illustration of culture transfer from feeder/intermediate container to the bulk starter tank is shown in Fig. 8.8 (see also Tamime, 1990; Bylund, 1995).

According to Bylund (1995), it is normally recommended that two tanks should be used in rotation; one contains ready-made starter for use and the other is for

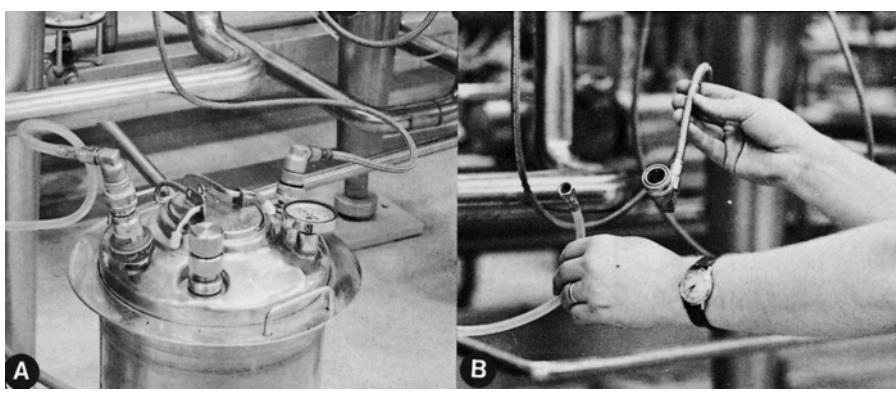

Fig. 8.10 Pipes, valves, connections and fittings for the feeder/intermediate culture container

A, Temperature dial, right hand pipe for air supply; the left hand plastic pipe is for outlet. B, Special valve fitments with quick release coupling; the pipe description is as (A).

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preparing starter for the following day. The specifications of the bulk starter tank could be summarised as follows:

- The tank is of an aseptic design (i.e. hermetically sealed and triple jacketed).
- It is capable of withstanding negative and positive pressures up to 30 and 100 kPa, respectively.
- The agitator is operated via a two-speed motor and the shaft of the agitator is double sealed.
- It is fitted with HEPA filters (see Fig. 8.8) which can be sterilised by steam at 140°C and a stationary pH meter designed to withstand the extreme temperature differences during the cleaning of the tank, preparation of the milk and production of the starter culture.

8.4.4 pH control systems

Bulk starter systems using pH control techniques were produced and developed for the following reasons:

- To overcome the drawbacks associated with BRM/BIM (see Section 8.4.5), including the cost of such media.
- To minimise daily fluctuations in acid development of the conventional cheese bulk starter (i.e. over-ripe or less active) that occur under commercial practice (see Pearce and Brice, 1973; Walker *et al.*, 1981).
- To produce concentrated starter cultures at high pH about 5 (i.e. reducing the cellular damage that may occur in certain starter cultures held for long duration at low pH) and, as a consequence, to require less culture for production.

Two methods are available for production of starter cultures using the pH control system: external pH control and internal pH control. To our knowledge these systems are not used for the production of yoghurt bulk starter cultures, but for further information see the reviews by Sinkoff and Bundus (1983), Thunell and Sandine (1985) and Tamime (1990).

8.4.5 Bacteriophage resistant/inhibitory medium (BRM/BIM)

BRM/BIM are also referred to as phage resistant or inhibitory medium (PRM/PIM) and the basic ingredients are milk solids, sugar, stimulatory compounds (yeast extracts, pancreatic extracts and/or hydrolysed cereal solids), phosphate–citrate buffer and chelating compounds (ammonium or sodium phosphates). The latter compounds are essential to bind the free calcium (Ca^{2+}) and magnesium (Mg^{2+}) ions in the growth medium, and in particular Ca^{2+} which is required by bacteriophages during their proliferation and replication. Such growth media have been formulated mainly for cheese starter cultures and to a very limited degree for *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*; furthermore, the data compiled by Tamime (1990) on BRM/BIM indicate that, except under certain conditions, they are not very effective. It is important to note that phosphates in the bulk starter milk adversely affect the growth of *L. delbrueckii* subsp. *bulgaricus* (see Chapter 6, Section 6.3.10). At the present time, therefore, BRM/BIM are not widely employed in the yoghurt industry and it is safe to conclude that although such an approach may result in success with the mesophilic lactic starter cultures, its application for

the production of phage-free, yoghurt bulk starter cultures is limited. However, another approach to control the effect of bacteriophages on yoghurt organisms is the addition of formic acid to the culture (Lembke *et al.*, 1987).

8.5 Conclusion

Since the 1950s, there have been many developments in the field of starter culture technology (i.e. preservation, maintenance and production). The ultimate objectives of this work were to secure the availability of different strains of yoghurt starter cultures for the dairy industry, to ensure the purity and activity of these culture(s), and to devise appropriate systems for their use in the production of bulk starters in a creamery. Mechanically protected starter tanks were developed primarily for the cheese industry in order to control the proliferation of phage during the production of bulk starter cultures, but in view of the fact that *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* are also vulnerable to bacteriophage attack (see Chapter 6), the same precautionary methods have been adopted in the yoghurt industry.

At present there is a growing tendency for yoghurt producers to use concentrated freeze-dried and frozen cultures for the production of bulk starters and/or yoghurt, especially when using bio starters or tailor-made blended starters to produce the desirable characteristics in yoghurt (e.g. mild or sharp taste, low or high in acetaldehyde and/or low or high viscosity). However, some of these cultures may pose a problem during culture transfer with some mechanically protected bulk starter systems. Rehydration of the freeze-dried culture in a sterile liquid or in the case of cultures packaged in cans (ring-pull type), the transfer of either the rehydrated or the thawed cultures to the bulk starter tanks, is carried out using a sterile hypodermic syringe, and hence it could be difficult to employ these cultures in conjunction with certain types of tank. Similar difficulties arise with the pelletted concentrated frozen cultures, where thawing prior to inoculation is not recommended, and with concentrated freeze-dried cultures, particularly when using the Lewis system. However, these difficulties will be readily overcome as starter culture technology progresses and the production of bulk starters within a creamery will no longer be required as DVI systems become more widely used.

8.6 References

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